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13. ABSTRACT *(Maximum 200 words)*

Cancer cells often contain disruptions in the transition from G1 phase to S phase. The retinoblastoma protein (pRb) is one negative regulator of cellular proliferation. Many breast cancers retain functional pRb and therefore must use other mechanisms to alleviate the tumor-suppressive function of pRb. One common mechanism to circumvent pRb function is amplification of the G1 phase cyclin dependent kinases (cdk), cdk4 and cdk6. Cyclin dependent kinase 4 and cdk6 are thought to have redundant functions as pRb regulators, despite an increasing body of evidence suggesting that certain tumor types specifically activate either cdk4 or cdk6. This implies that either the function or the regulation of these kinases may be different in different cell types. Data presented here show that cdk4 and cdk6 have different abilities to drive the cell cycle from G1 into S phase in the osteosarcoma cell line, U2OS. Understanding differential regulation of these two kinases and their potentially different activation in response to mitogenic signals may eventually provide a mechanism to therapeutically interrupt kinase activity. Ability to interrupt kinase activity separate from cyclin D1 association may prove an important intervention in breast cancer.

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INTRODUCTION:

Cancer is characterized in part by a loss of cellular growth control. An important regulator of cellular growth is the retinoblastoma protein (pRb), which acts as a negative regulator of cellular proliferation (For review see (Weinberg 1995)). This protein has been found to be inactivated in several tumor types and the vast majority of human tumors contain mutations, amplifications or deletions that result in inactivation of pRb. Deregulation of the "pRb pathway" can result from mutations of both positive and negative regulators of pRb. For example, in breast cancers cyclin D1 is often amplified. Cyclin D1 activates cyclin dependent kinase 4 (cdk4) and cdk6, to phosphorylate pRb and inactivate its cell cycle inhibitory function. The D-cyclin kinases, cdk4 and cdk6 are also often amplified in human cancers and inhibitors of kinase function are often deleted in tumors.

The cyclin D-dependent kinases play a pivotal role linking growth regulatory signals to cell division. The activity of these kinases is very tightly controlled through periodic synthesis and destruction of the cyclin subunits, by phosphorylation and dephosphorylation of the kinase subunit, and through complex formation with two families of cyclin-dependent kinase inhibitors (CKIs) (Morgan 1995). The CIP/KIP family of inhibitors includes p21, p27 and p57, which associate with several different cyclin/cdk complexes (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Polyak et al. 1994; Toyoshima and Hunter 1994). These proteins may act as stimulators of cdk activity as well as inhibitors, since p21 has been shown to both activate and inactivate cyclin/cdk complexes, perhaps dependent on stoichiometry (Zhang et al. 1994; LaBaer et al. 1997). In contrast to the CIP/KIP family, the INK family of CKIs ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) specifically inhibit the activities of cdk4 and cdk6 by binding directly to the kinase subunit, disallowing association with the activating cyclin D subunit (for review see (Peter and Herskowitz 1994)).

The purpose of the research presented here is to investigate the pathways by which cdk4 and cdk6 stimulate unregulated cell division and the mechanism by which these kinases are activated. Numerous reports have shown that increased expression and protein levels of cdk4 and cyclin D proteins are found in breast cancer. Results summarized here suggest novel mechanisms of regulation of cdk4 and cdk6 and also indicate functional differences between these two kinases.

BODY

Experiments outlined in Aim 1 and summarized in the Statement of Work describe experiments to determine the contribution of inhibitor binding (p15, p16, p18) to kinase function as measured by pRb phosphorylation. As outlined in the Statement of Work, SAOS-2 cells were transfected with mutant forms of cdk4 and cdk6 to determine the effect of these proteins on pRb phosphorylation by immunoprecipitation. In addition, this aim described experiments designed to test the cdk6 mutants for their ability to bind cyclin D1 and the INK family of inhibitors. These experiments were scheduled in the statement of work to be started early in the research outline (Month 1-3 and Month 3-18, respectively).

Results of studies of pRb phosphorylation of immunoprecipitated SAOS-2 extracts revealed the following result. Transfected cdk6NFG, although determined to be kinase inactive in *in vitro* kinase assays, resulted in phosphorylation of pRb *in vivo* due to titration of p16. SAOS-2 cells express high levels of p16 and when cdk6NFG is expressed to high levels in these cells, the cdk6NFG protein binds p16, freeing endogenous cdk6(wt) from this inhibitor, presumably allowing complex formation with cyclin D1 and resulting in pRb phosphorylation *in vivo*. In contrast, cdk6R31CNFG was determined to be unable to phosphorylate pRb *in vivo* since it has no endogenous kinase function and is unable to titrate inhibitors away from the endogenous cdk6 protein.

In addition to these studies, experiments were also undertaken to determine the ability of the cdk6 mutants to bind cyclin D1 and the INK family of inhibitors. These immunoprecipitation studies were undertaken in U2OS cells, an osteosarcoma cell line which contains detectable levels of cyclin D1 as well as the inhibitor p18, and are deleted for p16. These studies demonstrated that the R31C mutation disrupted binding of cdk6 to p18 in the context of both the single (cdk6R31C) and double (cdk6R31CNFG) mutant. Furthermore, it was found that this mutation did not disrupt the binding of these mutant proteins to cyclin D1 nor to the CIP/KIP family inhibitor p27.

Concomitant with immunoprecipitation studies, cell cycle analysis of transfected U2OS cells were also undertaken. These studies revealed differences in the cell cycle profiles of transfected cdk4 and cdk6 cells. This observation led to further investigation of potential functional differences between cdk4 and cdk6. These studies resulted in a manuscript that is currently being submitted for

publication. This manuscript entitled "A cdk6-specific acceleration of G1 phase transit dependent on subcellular localization and $p18^{INK4C}$ interaction" is attached in the appendices. This manuscript is not yet accepted for publication and is therefore proprietary data and is marked as such. The data in this manuscript demonstrates a specific function for cdk6 in accelerating G1 phase of transfected U2OS cells but no corresponding G1 acceleration in cdk4 transfected cells. Furthermore, these data suggest a positive role for the INK family of inhibitors in cdk6 function. This finding will be further investigated to determine if activation of cdk6 by mitogenic signals is transmitted through the inhibitor protein, p18.

To further investigate both the potentially positive and negative functions of p18 in cell cycle progression, studies may be undertaken in human breast cancer cell lines. One breast cancer cell line, BT-20, contains a mutation in p18 that results in an inability of this p18 protein to bind cdk6. These cell lines may allow us to determine the contribution of cdk6 and p18 to pRb phosphorylation function in these breast cancer lines. As outlined in Aim 2 and Aim 3, studies investigating the contribution of ras and the CIP/KIP inhibitor, p21 in cdk4 and cdk6 function will be further investigated. If p18 is involved in the signal transduction pathway to cdk6 (cdk4) activation, ras studies may help us to determine the signaling pathway involved in this activation. The studies outlined in Aim 2 may also allow us to investigate the contribution of cyclin D1 levels in the activation of cdk4 and cdk6.

CONCLUSIONS

The studies presented here have shown that cdk4 and cdk6 have separable functions when ectopically expressed in U2OS cells. These findings are the first indication that cdk4 and cdk6 may have distinct functions and possibly distinct methods of regulatory control. Independent regulation of cdk4 and cdk6 could allow these kinases to respond to separate mitogenic signals in varied cell types or developmental stages. Thus, certain types of tumors may specifically amplify either cdk4 or cdk6. For instance, melanoma has been found to preferentially hyperactivate cdk4 while squamous cell carcinomas have been shown to amplify cdk6 activity (Wolfel et al. 1995; Timmermann et al. 1997). Since the activating partner of cdk4 and cdk6 (cyclin D1) is amplified in breast cancers, understanding other regulatory steps in cdk4 and cdk6 activation may allow intervention to inactivate these kinases despite high levels of cyclin D1 in these tumors.

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APPENDICES

Manuscript "A cdk6-specific acceleration of G1 phase transit dependent on subcellular localization and $p18^{INK4C}$ interaction".

PROPRIETARY DATA

A cdk6-specific acceleration of G1 phase transit dependent on subcellular localization and p18^{INK4C} interaction

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Running title: cdk6 specific G1 acceleration

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Abstract

Cyclin dependent kinase (cdk) 4 and cdk6 are thought to have redundant functions as pRb regulators, despite an increasing body of evidence suggesting that certain tumor types specifically activate either cdk4 or cdk6. This implies that either the function or the regulation of these kinases is different in different cell types. Consistent with this, results presented here show that cdk4 and cdk6 have different abilities to drive the cell cycle from G1 into S phase in U2OS cells. Ectopic expression of cdk6 results in an increased percentage of S-phase cells due to a shortened G1 phase, but similar expression of cdk4 does not produce this effect. These studies also revealed a novel positive role for $p18^{INK4c}$ in this cdk6-specific cell cycle progression. A mutant form of cdk6 (cdk6R31C) that cannot interact with $p18^{INK4c}$ was unable to accelerate G1 transit, apparently due to a defect in nuclear accumulation. Because $p18^{INK4c}$ can compete with cytoplasmically-localized cdc37 for binding to wildtype cdk6 , but not cdk6R31C, $p18^{INK4c}$ may play a critical role in generating functional, nuclear cdk6/cyclin D complexes when expressed at low levels, and may be inhibitory only upon high level expression. This role for $p18^{INK4c}$ in cdk6 activation coupled with the cdk6-specific G1 acceleration observed in these experiments suggests that cdk6 and cdk4 may respond to different mitogenic stimuli in the same cell and thus play unique roles in the G1-to-S-phase transition.

Introduction

In mammalian cells the regulation of cell division is tightly controlled through a series of checkpoints within the cell cycle including the restriction point in late G1 phase, a checkpoint that determines commitment to DNA replication. The restriction point may be viewed as the culmination of activation of G1 cyclin-dependent kinases, enzymes that govern cell cycle progression through phosphorylation of key regulatory substrates. Specifically, the cyclin D proteins and their associated kinases, cyclin dependent kinase (cdk) 4 and cdk6, function early in G1 phase of the cell cycle to link growth regulatory signals to the control of cell division. Both cdk4 and cdk6 can be activated by all three D-type cyclins (cyclins D1, D2 and D3) and are thought to have redundant functions as positive effectors of G1 progression (Matsushime et al. 1992; Meyerson et al. 1992; Matsushime et al. 1994; Meyerson and Harlow 1994). Activation of cdk4 and cdk6 allows progression from G1 phase to the start of DNA synthesis in normal eukaryotic cells by phosphorylating and inactivating the retinoblastoma protein (pRb). This initial modification of pRb by cdk4/cdk6-dependent phosphorylation may be followed by further phosphorylation by cyclin E/cdk2 complexes and ultimately relieves repression of E2F-dependent promoters, allowing the transcription of S-phase genes and the onset of DNA replication (for review see (Weinberg 1995)).

Because the cyclin D-dependent kinases play a pivotal role linking growth regulatory signals to cell division, activity of these kinases is very tightly controlled. Kinase activity is regulated by the periodic synthesis and destruction of the cyclin subunits, by phosphorylation and dephosphorylation of the kinase subunit, and through complex formation with two families of cyclin-dependent kinase inhibitors (CKIs) (Morgan 1995). The CIP/KIP family of inhibitors includes p21, p27 and p57, which associate with several different cyclin/cdk

complexes (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Polyak et al. 1994; Toyoshima and Hunter 1994). These proteins may act as stimulators of cdk activity as well as inhibitors, since p21 has been shown to both activate and inactivate cyclin/cdk complexes, perhaps dependent on stoichiometry (Zhang et al. 1994; LaBaer et al. 1997). In contrast to the CIP/KIP family, the INK family of CKIs ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) specifically inhibit the activities of cdk4 and cdk6 by binding directly to the kinase subunit, disallowing association with the activating cyclin D subunit (for review see (Peter and Herskowitz 1994)).

Aberrant cell proliferation and tumorigenesis can result from deregulated activity of cdk4 and/or cdk6 with subsequent, inappropriate inactivation of pRb in several tissue types. This increased kinase activity can result from overexpression of the regulatory subunit, cyclin D1, and also from amplification of the kinase-encoding gene. In addition, deletion or inactivation of the gene encoding $p16^{INK4a}$ frequently leads to dysregulated cdk4/cdk6 activity in human tumors, as do mutations in cdk4 that prevent its association with $p16^{INK4a}$ (Motokura et al. 1991; Khatib et al. 1993; Kamb et al. 1994; Nobori et al. 1994; Tsujimoto et al. 1994; Ladanyi et al. 1995; Wölfel et al. 1995; Costello et al. 1997). In most of these cases, tumors containing hyperactivated cdk4 or cdk6 retain intact RB alleles, suggesting that such kinase activations render pRb unable to control proliferation. These findings indicate that deregulated cdk4 and cdk6 activity can substitute for RB mutations, and define the "pRb pathway" of genetic events that have the identical phenotypic consequence of pRb inactivation and inappropriate proliferation.

Inconsistent with the apparent functional redundancy of each of these genetic events in the pRB pathway in cancer is the fact that many cells express two or three D-type cyclins and both cdk4 and cdk6. Furthermore, varying

tumor types have been found to preferentially alter certain components of this pathway and to specifically increase activity of either cdk4 or cdk6, but not both. For instance, cdk4 seems to be specifically targeted in melanoma (Wölfel et al. 1995; Zuo et al. 1996) while increased cdk6 activity was found in squamous cell carcinomas (Timmermann et al. 1997) and neuroblastomas (Easton et al. 1998) without alteration of cdk4 activity. These findings suggest that cdk4 and cdk6 may not be entirely redundant in all cells and may be individually targeted by distinct cell-type specific mitogenic signals directing their temporal or substrate-specific activities.

To further investigate potentially distinct functions of cdk4 and cdk6 in growth-promotion, we investigated the role of these kinases in G1 progression in the osteosarcoma cell line, U2OS. Cells transfected with cdk6, but not cdk4, demonstrated accelerated transit through the G1 phase of the cell cycle. Thus, these experiments recapitulate the preference for cdk6 activation seen in the cancers described above, and suggest that cdk4 and cdk6 can be differentially active in the same cell. This specific effect of cdk6 on cell cycle progression was dependent on the INK4 binding domain, since a cdk6 mutant (R31C) unable to associate with INK4 proteins did not show G1 acceleration. In addition, this mutant protein failed to accumulate in the nucleus, suggesting that nuclear localization and function of cdk6 is dependent on INK4 interaction. U2OS cells, like many other tumor cells, express $p18^{INK4c}$ but not $p16^{INK4a}$, suggesting that interaction of cdk6 with $p18^{INK4c}$ is involved in the generation of a functional, nuclear cyclin/cdk6 complex. Consistent with this, we observed that elevated expression of $p18^{INK4c}$ prevented interaction of wildtype cdk6 with the cytoplasmic protein cdc37 (a protein targeting subunit of Hsp90), (Stepanova et al. 1996; Lamphere et al. 1997), but was ineffective at dissolving complexes of cdc37 and cdk6R31C. In a manner analagous to that proposed for p21,

$p18^{INK4c}$ (and perhaps other INK4 proteins) may function through cdk6 as a positive effector of cell cycle progression when expressed at low levels, and may adopt an inhibitory role only upon elevated expression in response to anti-mitogenic cues.

Results

Cdk6, but not cdk4, causes increased S-phase of transfected cell populations.

To determine the effects of ectopic expression of cdk4 and cdk6 on cell cycle progression, fluorescence-activated cell sorting (FACS) of transfected U2OS cells was performed using CD20 to identify transfected cells. These human osteosarcoma cells produce wild-type pRb but lack $p16^{INK4a}$, a defect that is thought to allow constitutive phosphorylation and inactivation of pRb. In light of this, it was surprising to observe that cells transfected with plasmid encoding the cdk6 cDNA consistently showed a higher percentage of S-phase cells than did vector transfected cells in the same experiment (Figure 1). Most importantly, ectopic expression of cdk4 was without effect in these experiments, demonstrating the increase in S-phase cells was specific to cdk6 (Figure 1). To confirm that this increase in DNA content measured by FACS truly reflected an increase in S-phase cells, transfected cell cultures were analyzed using bromodeoxyuridine (BrdU) incorporation as a measure of S-phase. U2OS cells transfected with pCMVpcdk4, pCMVcdk6, or pCMVvector were pulsed with BrdU for 60 minutes, fixed and subjected to indirect immunofluorescence using both anti-BrdU and anti-cdk4 or anti-cdk6 antibodies. Transfected cells were identified as those that demonstrated intense fluorescence with anti-cdk antibodies and were scored as either BrdU-positive or BrdU-negative. The results of at least two independent transfections in which a total of at least 500 kinase positive cells were counted, are presented in Table 1. These data agreed

with our original observation that cells transfected with cdk6 showed a statistically significant ($p<.05$) increase in the percentage of S-phase population as compared to cdk4-transfected or vector-transfected cells.

The observed increase in S-phase cells could result from either an S-phase cell cycle block or decreased transit time through G1 or G2/M. To distinguish between an S-phase delay and acceleration through other cell cycle compartments, transfected cells were treated with the mitotic inhibitor nocodazole. In the presence of nocodazole, an S-phase delay caused by cdk6 would reduce the number of cells able to enter G2/M. However, if the increased S-phase population was due to a shortening of G1 phase (or G2/M phase) the cells would arrest in mitosis under nocodazole treatment. At 24 hours after removal of DNA precipitates, parallel sets of transfected U2OS cells were either harvested or were treated with nocodazole for 18 hours. Controls for nocodazole arrest demonstrated that untransfected cells accumulated in mitosis while p16 transfected cells maintained a G1 phase peak as expected with a G1-arresting inhibitor (Figure 2). In the same experiment, cdk6-transfected cells did not maintain an S-phase peak but accumulated in mitosis in the presence of nocodazole, indicating that the S-phase increase seen by FACS and BrdU incorporation studies in the absence of nocodazole was due to a decrease in G1 or G2/M transit time.

cdk6 shortens the G1 interval of transfected U2OS cells.

To determine if the increase in S phase cells in cdk6-transfected cultures was due to a decrease in G1 transit time, U2OS cells were synchronized and BrdU incorporation was studied as cells traversed G1 and entered S-phase. U2OS cells transfected with cdk4 or cdk6 plasmids were synchronized by nocodazole followed by shaking to isolate the mitotic cells, and were replated in

the absence of nocodazole. At four separate timepoints after mitotic shake, BrdU was incorporated for 60 mins prior to the 5, 7, 8, and 10 hr timepoints and incorporation was measured by indirect immunofluorescence. The results of this experiment are shown in figure 3A. By this method, cdk6 and cdk4 transfected cells show approximately equal BrdU incorporation at 5 hours but at all later time points approximately 40% more cdk6 transfected cells incorporated BrdU than did cdk4 transfected cells. Thus, this experiment suggested that cdk6 expression accelerated S-phase entry as compared to cdk4 expression. To confirm and extend this result, FACS analysis was performed on synchronized cell populations. Transfected U2OS cells were treated with nocodazole for 18 hours followed by shaking and replating in nocodazole-free medium as described above. The cells were then harvested at 4 hours and 8 hours after mitotic shake and DNA profiles of CD20 positive cells were obtained by FACS as shown in Figure 3B. At 4 hours after mitotic shake, cdk6-transfected cells showed a synchronized DNA profile indistinguishable from that of vector transfected cells. Interestingly, at 8 hours post mitotic shake, cdk6 transfected cells showed a shift toward S-phase (increased DNA content) as compared with vector transfected cells. Together, these experiments suggest that cdk6-transfected, but not cdk4-transfected cells pass through G1 phase faster than vector-transfected cells, demonstrating a cdk6-specific acceleration of G1 transit in these U2OS cell cultures.

Biochemical characterization of cdk6 mutants.

The acceleration of G1 phase caused by ectopic expression of cdk6 could be the result of direct catalytic activity of the introduced kinase subunit phosphorylating substrates like pRb to shorten G1 phase. Alternatively, excess kinase subunits could titrate inhibitory proteins to allow activation of other cdks

and concomitant cell cycle advance. Titration of inhibitory proteins has been observed to occur upon introduction of both functional and nonfunctional kinases in another system, apparently through titration of p21 (Latham et al. 1996), and cyclin D/cdk4(6) complexes have been suggested to sequester p27 in the absence of anti-mitogenic signals (Reynisdottir et al. 1995; Reynisdottir and Massague 1997). In an effort to determine the properties of cdk6 required to accelerate G1 progression in U2OS cells, a series of cdk6 mutants that are compromised in their ability to bind to INK4 protein (cdk6R31C), hydrolyze ATP (cdk6NFG) (van den Heuvel and Harlow 1993), or both (cdk6R31CNFG) were used in cell cycle analyses. The biochemical characterization of these mutant proteins is presented in Figure 4. Importantly, all cdk6 mutants consistently showed approximately equal protein levels upon transfection into U2OS cells as shown by immunoblot in Figure 4A. Consistent with the predicted result, we found that the R31C mutation prevented interaction with $p16^{INK4a}$ (data not shown). Immunoprecipitations of transfected U2OS lysates demonstrated that cdk6R31C failed to bind detectably to $p18^{INK4c}$ (Figure 4B), as has been previously observed in breast cancer cell lines (Lapointe et al. 1996). This interaction is particularly relevant to U2OS cells since these cells lack $p16^{INK4a}$, yet express detectable levels of $p18^{INK4c}$ bound to cdk6 (data not shown). Disruption of INK4 binding occurred whether the mutation was present alone (cdk6R31C), or in combination (cdk6R31CNFG) with the catalytically inactive mutation (Figure 4B). Importantly, the R31C mutation does not disrupt the ability of cdk6 to bind cyclin D1 or p27 in immunoprecipitations of transfected U2OS extracts (Figure 4C, 4D). In these experiments, 200 μg (Cyclin D1 binding) or 400 μg (p18 and p27 binding) of transfected U2OS cell lysates were immunoprecipitated with anti-cdk6 polyclonal antibody and immunoblots were performed with the appropriate antisera. The same blot was

then stripped and reprobed with anti-cdk6 antibody to ensure equal levels of cdk6 protein were compared in binding studies. Thus, the cdk6R31C mutation that corresponds to the tumor-derived cdk4R24C mutation (Wölfel et al. 1995) specifically disrupts cdk6 binding to INK4 proteins without altering interaction with other known cdk6 partners.

To ensure that the R31C mutant form of cdk6 retained catalytic activity, the cdk6 mutants were also examined for kinase activity in transfected SAOS-2 cells. When co-transfected with cyclin D1 (SAOS-2 cells contain low levels of endogenous cyclin D1) and immunoprecipitated with antibody to the HA tag, HAcdk6 phosphorylated the C-terminal GST-Rb substrate. Conversely, both cdk6 mutants containing the kinase inactivating mutation (cdk6NFG, cdk6R31CNFG) showed no kinase activity above vector-transfected background. Significantly, cdk6R31C-transfected extracts reproducibly showed *in vitro* kinase activity greater than that observed with wildtype cdk6 extracts, as expected for a mutant that can evade the $p16^{INK4a}$ present at high level in SAOS-2 cells. Anti-HA immunoblots of these extracts confirmed that the level of cdk6R31C was at or below the level of wildtype cdk6 protein (not shown).

Thus, the cdk6R31C mutation disrupts INK4 protein binding but did not disrupt intrinsic catalytic activity of this cdk6 protein, similar to studies demonstrating retention of kinase activity by the $p16^{INK4a}$-binding defective mutant of cdk4, cdk4R24C (Wölfel et al. 1995). These reagents are thus ideal for assessing the potential roles of catalytic activity and INK4 titration in the cdk6-mediated acceleration of G1 phase in transfected U2OS cells.

cdk6 mutants do not accelerate G1 phase.

The cdk6 mutants described above were used to further examine cdk6 function in G1 acceleration of U2OS cells. To test the ability of mutant forms of

cdk6 to decrease G1 transit time, FACS analysis of transfected cells was performed at 24 hours post transfection as described above for the wild-type protein. The results of these FACS studies indicated that the kinase inactive mutant, cdk6NFG, showed an increase in G1 population as compared to the vector control. Nocodazole treatment suggested that this G1 increase was likely due to a G1 delay, since a significant G1 fraction persisted in nocodazole arrested cells. Thus, catalytic activity and not inhibitor titration appears to be required for the G1 acceleration observed with wildtype cdk6 (Figure 5A), since cdk6NFG was incapable of increasing the S phase fraction of transfected cells, but is fully capable of inhibitor interaction.

Because the result above suggests that kinase activity intrinsic to cdk6 is key to accelerating G1 phase in transfected U2OS cells, the cdk6R31C mutant was expected to give an increase in S phase cells equal to or greater than that conferred by wild-type cdk6, given the ability of cdk6R31C to phosphorylate pRb and avoid interaction with INK4 proteins. Surprisingly, however, cdk6R31C did not show the G1 acceleration typical of wildtype cdk6, as might be expected if $p18^{INK4c}$ acts to limit cdk6 activity in these cells (Figure 5A). Consistent with these FACS data generated from an asynchronous population, nocodazole treated cells also showed no enhancement of S-phase entry upon transfection with cdk6R31C (Figure 3B). Interestingly, when the R31C mutation was combined with the NFG mutation, the double mutant (cdk6R31CNFG) failed to show the G1 increase seen with cdk6NFG. Thus, the R31C mutation nullified both the G1 acceleration function of cdk6wt and the G1 delay function of cdk6NFG (Figure 5A).

BrdU incorporation was also used to measure S-phase entry by the cdk6 mutants. In these experiments BrdU was added to transfected U2OS cells 4 hours after mitotic shake and BrdU incorporation was measured at 5, 7, and 10

hours after mitotic shake. The results of these experiments in which cumulative BrdU incorporation was measured are presented in figure 5B. In agreement with the FACS results, both cdk6R31C (41%) and cdk6R31CNFG (38%) show significantly fewer cells in S-phase than did cdk6wt (60%) at 10 hours after release from mitotic block. The values for the cdk6R31C and cdk6R31CNFG mutants were similar to that of the vector transfected control (39%). In agreement with the FACS studies, cdk6NFG showed a greatly decreased percent of cells in S-phase at all timepoints measured (27% at 10 hrs). Thus, intrinsic kinase activity appears to be necessary but not sufficient for the increase in S-phase population caused by cdk6wt since cdk6R31C, which has demonstrated in vitro kinase ability, cannot accelerate G1 phase. Further, the R31C mutation in cdk6R31CNFG nullifies the S-phase inhibitory effect seen after introduction of cdk6NFG, suggesting that the R31 residue of cdk6 is critical to the formation of functional cyclin/cdk complexes.

Cell cycle effects correlate with nuclear localization.

Previous studies have shown that the subcellular localization of cdk4 may influence its interaction with the CIP and INK family of inhibitors (Reynisdottir and Massague 1997) and J. Koh, personal communication). In addition, both cdk4 and cdk6 have been observed to localize to the cytoplasm in a variety of cell types (Stepanova et al. 1996; Nagasawa et al. 1997; Mahony et al. 1998). Thus, we wished to test the hypothesis that the inability of the cdk6 mutants to accelerate G1 phase may be in part due to differential localization within the cell as ascertained by indirect immunofluorescence. Transfected U2OS cells were synchronized using nocodazole and coverslips were removed and fixed at timepoints after mitotic shake. Repeatedly, the mutant forms of cdk6 that failed to bind $p18^{INK4c}$ (cdk6R31C and cdk6R31CNFG) demonstrated greatly

decreased nuclear staining as compared to cdk6wt and cdk6NFG at 8-10 hours after mitotic shake (Figure 6). These results were repeated in at least 3 separate transfections and in multiple immunofluorescence staining studies with both polyclonal and monoclonal antibodies (Figure 6).

The decrease in nuclear staining observed in cdk6R31C and cdk6R31CNFG transfectants was also observed in asynchronous populations at 24 hours after removal of DNA precipitates but in these populations, the percent of cdk6R31C and cdk6R31CNFG mutants with predominantly cytoplasmic staining was lower than the percent seen in a synchronous population (as shown in figure 6), suggesting that the cellular localization of these kinases is cell cycle regulated. The striking decrease in nuclear staining of cdk6R31C and cdk6R31CNFG was apparent in coverslips fixed by two distinct fixing methods (70% ethanol or methanol/acetone) and with multiple antibodies, including a polyclonal rabbit antiserum (Meyerson and Harlow 1994) and a commercially available mouse monoclonal antibody (Figure 6B).

Together, these studies demonstrate that the R31C mutation affects compartmentalization of cdk6 as well as the ability to interact with INK4 proteins. R31C mutants showed a remarkable decrease in nuclear staining particularly at timepoints predicted to be at or near the G1/S boundary. This difference in compartmentalization directly correlated with the inability of the same mutants to accelerate G1 phase of the cell cycle and suggested a role for INK4 protein association in the generation of functional, nuclear cdk6 complexes.

$p18^{INK4c}$ competes cdc37 from cdk6.

Published studies have demonstrated that cdk4 and cdk6 exist in complex with cdc37 and hsp90 and that $p16^{INK4a}$ competes with cdc37 for cdk4 binding

(Dai et al. 1996; Stepanova et al. 1996; Lamphere et al. 1997). Since the cdc37/cdk complex is localized to the cytoplasm, we surmised that an inability to bind $p18^{INK4c}$ may prevent cdk6R31C from dissociating from cdc37, and this persistent interaction with cdc37 may explain the predominantly cytoplasmic localization and inactivity of cdk6R31C. To determine if $p18^{INK4c}$ could compete with cdc37 for cdk6 binding, we immunoprecipitated cdk6 and cdk6R31C transfected U2OS cells in the presence and absence of co-transfected $p18^{INK4c}$. Transfected U2OS cell lysates were immunoprecipitated with cdk6 polyclonal antibody and immunoblotted with cdc37 antibody and cdk6 antibody. As shown in figure 7, cdk6wt produced in the presence of excess $p18^{INK4c}$ shows a marked reduction in binding to cdc37 as compared to that seen in cells transfected with cdk6 alone, indicating that transfected $p18^{INK4c}$ was indeed competing with endogenous cdc37 for cdk6 binding. In contrast, no decrease in cdc37 was observed in immunoprecipitations of lysates derived from cells co-transfected with cdk6R31C and $p18^{INK4c}$ compared to those transfected with cdk6R31C alone. Similarly, a competition between $p18^{INK4c}$ and cdc37 for cdk6 was also observed with transfected pCMVcdk6NFG, but not with cdk6R31CNFG (data not shown). Together, these data indicate that $p18^{INK4c}$ competes with cdc37 for binding to cdk6, and importantly, this competition does not occur with a $p18^{INK4c}$-binding defective cdk6 protein, suggesting that $p18^{INK4c}$ binding and cdc37 binding are mutually exclusive events. Thus, the predominantly cytoplasmic localization of cdk6R31C and its inability to accelerate G1 phase are likely due to cytoplasmic retention of cdk6R31C by cdc37.

Discussion

The D-cyclin-dependent kinases cdk4 and cdk6 share pRb as their only proven physiological substrate and both can act as oncogenes in human tumors that retain pRb, suggesting redundancy of function. Nevertheless, the fact that many cells express both cdk4 and cdk6, coupled with the observation that some tumor types specifically activate only one of these kinases, suggests that each kinase may play a unique role in cell cycle progression. The results presented here demonstrate cdk4 and cdk6 have separable functions when ectopically expressed in U2OS cells. Expression of cdk6, but not cdk4, resulted in increased S-phase as measured by FACS and BrdU incorporation. Nocodazole and mitotic release studies indicate that the observed increase in S-phase cells resulted from decreased transit time through G1 phase of the cell cycle. Surprisingly, the accelerated G1 phase observed with cdk6 overexpression does not require co-transfection of the kinase activating partner, cyclin D. G1 acceleration by cdk6 in the absence of increased cyclin D is consistent with a model in which the supply of cyclin D is not the only rate-limiting step in kinase activation. Indeed, cyclin D1 levels are, surprisingly, quite stable across the cell cycle in many proliferating cells unlike cyclins A, E, and B. Thus, while cyclin D complex formation is obviously a critical step in kinase activation, it may not be the rate limiting step in cultures of proliferating cells.

The availability of the kinase may be particularly limiting for cyclin D1/cdk4 (cdk6) complexes, since the INK4 family of inhibitors act as competitors with cyclin D for cdk4 and cdk6 binding. Thus, $p16^{INK4a}$-nonbinding mutants of cdk4 are oncogenic by virtue of their insensitivity to growth arrest (senescence) signals presumably transduced through $p16^{INK4a}$ (Hara et al. 1996; Serrano et al. 1997; McConnell et al. 1998; Wölfel et al. 1995). In sharp contrast, our results indicate that an analogous mutation of cdk6 that disallowed INK4 binding disrupted the ability of cdk6 to accelerate G1

phase. Because this mutation, R31C, does not disturb other properties of cdk6 such as cyclin D1 or p27 binding or *in vitro* kinase activity, we propose that binding of at least one member of the INK4 family may be required for production of functional cyclin D/cdk6 complexes in U2OS cells.

Published reports indicate that an additional level of regulation of cdk4 and cdk6 may be subcellular localization (LaBaer et al. 1997; Reynisdottir and Massague 1997). Consistent with these studies, data shown here demonstrate that cdk6wt localizes to both the nucleus and the cytoplasm while cdk6R31C preferentially localizes to the cytoplasm. U2OS cells released from nocodazole arrest show a striking lack of cdk6R31C and cdk6R31CNFG protein in the nucleus in late G1 phase. Recently it has been shown that cytoplasmic cdk4 and cdk6 exist primarily in inactive complexes with cdc37 and hsp90 or (in T cells) with $p19^{INK4d}$ (Stepanova et al. 1996; Mahony et al. 1998). In light of this, the localization pattern of the cdk6R31C protein presents an apparent paradox. Why is an INK4-binding defective cdk6 protein preferentially localized in the cytoplasm if the major function of the cytoplasmically-localized INK4 protein (in this case, specifically $p18^{INK4c}$) is to anchor kinases in an inactive state? This paradox can be explained by results in figure 7 that demonstrate that $p18^{INK4c}$ competes cdk6 from cdc37, a known cytoplasmic protein. Since $p18^{INK4c}$ is unable to compete cdk6R31C away from cdc37, the R31C mutant protein is apparently preferentially retained in the cdc37/hsp90 complex. Maintenance of the cdk6R31C mutant in the cdc37 complex predicts a persistence of cytoplasmic localization and a functionally inactive kinase. In fact, this is precisely the phenotype observed with cdk6R31C: An increased cytoplasmic retention (Figure 6) and a loss of function in either G1 acceleration (cdk6wt) or G1 retardation (cdk6NFG) (Figure 5), despite greater than wildtype catalytic activity of cdk6R31C in *in vitro* kinase assays (Figure 4E).

The competition of $p18^{INK4c}$ with cdc37 for cdk6 binding is consistent with results of Lamphere et. al., who demonstrated that $p16^{INK4a}$ competes with cdc37 for binding to cdk4 in insect lysates (Lamphere et al. 1997). Further, gel filtration studies have shown that inactive cdk6 exists transiently in a complex with cdc37/hsp90 but, more stably, in a complex with $p19^{INK4d}$ (Mahony et al. 1998). To reconcile the biological properties of cdk6R31C with the biochemical data described above, we propose a model in which $p18^{INK4c}$ is obligatorily involved in the liberation of cdk6 from the cdc37 complex and retains the kinase in the cytoplasm in an inactive state (Figure 8). Upon the transduction of an appropriate signal, cdk6 is released from the INK4 complex and translocated to the nucleus, possibly in complex with cyclin D. This model, in which subcellular compartmentalization of cdk6 is controlled by competing interactions with cdc37, $p18^{INK4c}$ and presumably D cyclins, raises the possibility that this is a regulated process responsive to proliferative signals. Indeed, subcellular compartmentalization is an important regulatory step in several cellular processes. Regulation through compartmentalization allows rapid response to signaling pathways since the previously-synthesized protein is present in an inactive, ready, state. For instance, the transcription factor NFkB is anchored in the cytoplasm by its inhibitor, IkB, until the appropriate signal is received and IkB is phosphorylated and degraded, releasing NFkB to translocate to the nucleus and activate specific target genes. Similarly, compartmentalization of the cyclin-dependent kinases may provide rapid response to mitogenic signals and link signal transduction pathways to the cell cycle machinery. This type of regulation might be especially important for the D-cyclin dependent kinases that initiate phosphorylation of pRb in response to mitogenic stimuli. Interestingly, the INK4 family members, including $p18^{INK4c}$, contain ankryin repeats typical of known cytoplasmic anchoring

proteins, such as IkB. It is possible that dissolution of the $p18^{INK4c}$/cdk6 complex in favor of cyclin D association involves degradation of $p18^{INK4c}$ and release of cdk6. Experiments designed to test this possibility are underway.

Importantly, our model does not predict that all INK4 family members act on cdk6 or on cdk4 in this same manner. Indeed, differences in the mechanism of activation of cdk4 and cdk6 could explain the unique ability of cdk6, but not cdk4, to promote S phase entry in transfected U2OS cells. For example, it has been shown that $p18^{INK4c}$ preferentially associates with cdk6 (Guan et al. 1994), leaving cdk4 regulation under control of other INK4 family members. Thus, while highly homologous, the INK4 proteins $p16^{INK4a}$ and $p18^{INK4c}$ may not have completely redundant functions and may allow separate mitogenic signaling pathways to end in activation of either cdk4 or cdk6, dependent on cell type or signal transduced. Supporting this notion, $p16^{INK4a}$, a known tumor suppressor protein, is inactivated in a great number of human cancers while $p18^{INK4c}$ gene inactivation was not found in studies of several primary tumors as well as several malignant cell lines of various origin (Ragione et al. 1996; Zariwala et al. 1996). These published data and those presented here may help to explain the surprising observation that the G1 inhibitor proteins, $p18^{INK4c}$ and $p19^{INK4d}$, are universally expressed in proliferating cells, yet are rarely mutated in tumors. In addition, these inhibitors are elevated in late G1 and early S phase (Hirai et al. 1995), an expression pattern unexpected of inhibitors involved in G1 arrest. This cell-cycle dependent expression of $p18^{INK4c}$ and $p19^{INK4d}$ may be a reflection of the role of these proteins as obligate intermediates in cdk6 activation that is predicted by our model.

Finally, it is important to note that our model does not preclude a role for $p18^{INK4c}$ and $p19^{INK4d}$ as inhibitors of cdk6 (and possibly cdk4) under appropriate circumstances. Indeed, elevated expression of these proteins, such as

has been observed upon differentiation (Franklin and Xiong 1996; Zindy et al. 1997; Phelps et al. 1998), could out compete D-cyclin association, particularly if formation of the D-cyclin/cdk6 complex requires further mitogenic signals as suggested above. Such a dual role for cdk inhibitors is not without precedent. It has been suggested that the CIP/KIP family of inhibitors can promote assembly of kinase complexes at lower concentrations and inhibit kinase activity at higher concentrations (Zhang et al. 1994; LaBaer et al. 1997), thus acting positively or negatively depending on the signals received by a particular cell. We propose that $p18^{INK4c}$ and $p19^{INK4d}$ have conceptually similar, dual roles in regulation of cdk6 activity. At low levels (subject to cell cycle regulation), these proteins may facilitate the assembly of active D-cyclin/cdk6 complexes by maintaining a cytoplasmic pool of "activatable" cdk6. However, when induced to high levels, for example in response to differentiation stimuli, these INK4 proteins may adopt the previously-suggested role of competitive inhibitor of cdk6 activation. This functional role for $p18^{INK4c}$ in release of cdk6 from an inactive cytoplasmic cdc37/hsp90 complex is supported by the observed inactivity of cdk6R31C and its apparent sequestration by cdc37 in the cytoplasm. We propose a model in which $p18^{INK4c}$ maintains cdk6 in a ready state awaiting the appropriate mitogenic signal to allow cdk6 to translocate to the nucleus in an active form. In this model, the multiplicity of INK4 family members and D-cyclin kinases could allow for a multitude of mitogenic signals to elicit cell type and developmentally specific proliferative responses.

Materials and Methods

Expression vectors, transfection procedures and cell lines. The kinase expression plasmids pCMVcdk4, pCMVcdk6 and HA-tagged cdk6, pCMVcdk6HA, pCMVcdk4nfg, pCMVcdk6NFG, the vector pCMVneobam and

the CD20-encoding plasmid, pCMVCD20, were kindly provided by Dr. Sander van den Heuvel (van den Heuvel and Harlow 1993). The vector containing these cDNA's pCMVBamNeo has been previously described (Baker et al. 1990). The osteosarcoma cell line, U2OS, was transiently transfected with 15 micrograms (μg) kinase-expressing plasmid plus 5 μgs pCMVCD20 (where appropriate) and sheared herring sperm DNA to a total of 30 μgs. U2OS cells were transfected by calcium phosphate precipitation essentially as described by Chen and Okayama (Chen and Okayama 1987). DNA precipitates remained on the cell monolayer for 17 hours and cells were harvested 24 hours after removal of DNA precipitates unless otherwise noted. U2OS cells were maintained in 10% fetal calf serum at 5% CO_2.

Analysis of cell cycle distribution. For fluorescence-activated cell sorting (FACS) experiments, transfected U2OS cells were harvested in PBS with .1% EDTA at 24 hours after removal of DNA precipitates. Cells were then stained with fluoresceine isothiocyanate (FITC) conjugated antibody to human CD20 (Pharmingen), ethanol fixed and stained with propidium iodide for DNA content. Cell cycle distribution was analyzed by flow cytometry of CD20-positive (FITC positive) cells using a Coulter Cytometer and the Multicycle DNA-analysis program. In FACS studies of nocodazole treated cells, nocodazole was added at 24 hours after removal of DNA precipitates to a final concentration of 100 ng/ml for 18 hours. In FACS studies following mitotic shake, U2OS cells were treated with 100 ng/ml nocodazole for 18 hours and mitotic fractions were harvested by gentle pipeting followed by centrifugation at 1000 rpm at 25°C for 5 mins. Cells were washed three times with media to remove nocodazole and replated in media containing 10% fetal calf serum. Cells were then harvested at time points indicated and prepared for flow cytometry as described above.

BrdU and immunofluorescence. In 5-bromo-2'-deoxy-uridine (BrdU) incorporation experiments nocodazole was added to 100 ng/ml approximately 5 hours after removal of DNA precipitates and remained on the cells for 18 hours. BrdU was added to a final concentration of 10 micromolar (μM) at time points indicated. Coverslips from BrdU experiments were fixed in 70% ethanol, 50 millimolar (mM) glycine, pH 2.0. After fixing, coverslips were incubated with monoclonal antibody to BrdU (Bohringer-Mannheim) and polyclonal peptide antibodies to cdk6 or cdk4 (Santa-Cruz, C-21 and C-22, respectively) for 60 mins at 37°C. Secondary antibody incubation was performed with rhodamine conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) and fluoresceine conjugated anti-mouse antibody (Bohringer-Mannheim) for 30 mins at 37°C. Coverslips were mounted in fluoromount to reduce quenching. For timecourses after nocodazole synchronization, mitotic shake was performed as described above and at indicated time points after re-plating coverslips were removed from the tissue culture dish and fixed. For immunofluorescence without BrdU incorporation, coverslips were stained in methanol followed by acetone. Indirect immunofluorescence was performed with antibodies as indicated above and in addition cdk6 polyclonal sera of Meyerson (Meyerson and Harlow 1994) and cdk6 monoclonal sera Ab-3 (Neomarkers, Fremont, CA) for 60 mins at 37°C. Secondary antibody staining was performed as described above or with fluoresceine-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). In relevant cases, cells were counterstained by Hoechst staining. All photography was performed on a Leica microscope with Sony digital imaging.

Biochemical assays. For immunoblot and immunoprecipitation experiments, 1.25 X 10^6 U2OS cells (30-50% confluent) were transfected as described above. Transfected cells were harvested at 24 hours after removal of DNA precipitates, washed twice with phosphate buffered saline and harvested in E1A lysis buffer (ELB) (250mM NaCl, 50mM Hepes pH 7.0, 5mM EDTA, .1%NP40). Extracts were incubated 20 mins on ice with mixing and then clarified by centrifugation for 20 mins at 4°C. Extracts were separated on polyacrylamide denaturing gels and transferred to supported nitrocellulose (Gibco BRL). Blots were developed using appropriate antisera as noted.

Immunoprecipitations were performed with 2µg polyclonal cdk6 antisera C-21. Transfected U2OS cells were lysed in ELB buffer as described above and 200-400 µg of extract (as noted) was immunoprecipitated for 60 mins at 4°C with mixing. Protein A-Sepharose swollen beads were added for an additional 30 mins. A total of 35µl of beads was used for 200-400µgs of cell lysate. Beads were washed 4 times with 1 ml of ELB. After immunoprecipitation, samples were separated on denaturing acrylamide gels and immunoblots performed as described above. Antibodies used include: p18, polyclonal N-20 1:3000 (Santa Cruz), p27, monoclonal antibody 1:2500 (Transduction Labs), cyclin D1 monoclonal DCS-6 1:200 (Neomarkers). In cdc37 experiments, 800µg of ELB lysate was immunoprecipitated with cdk6 C-21 antibody for 60 mins at 4°C and 50µl of swollen protein A sepharose beads were added for an additional 30 mins. Immunoprecipitations were washed four times with 1ml ELB, separated on denaturing acrylamide gels and immunoblotted with antibody to cdc37 (Stepanova et al. 1996) and commercially available polyclonal cdc37 antibody (Neomarkers).

For kinase assays, SAOS-2 cells at 80% confluency were transfected with 10µgs pCMVD1 and 10µgs pCMVcdk6HA or cdk6mutantHA in the pCMV

vector by calcium phosphate as described above. DNA precipitates remained on cells 10 hours and were harvested 36 hours after removal of DNA precipitates. Cells were harvested in D-IP Kinase Buffer (50mM Hepes pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, .1% Tween 20, 10% glycerol with protease inhibitors aprotinin, leupeptin and pefablock and phosphatase inhibitors sodium orthovanadate (100μM), sodium flouride (10mM), and betaglycerophosphate (10mM) and incubated on ice for 20 mins with gentle mixing. Lysates were clarified by centrifugation at 4°C for 10 mins. 100ul of 12CA5 antibody was preincubated with 30μl swollen protein A sepharose beads at least 1 hour at 4°C with mixing. 100μg of cell lysate was added for an additional hour at 4°C with mixing. Beads were washed 3 times with D-IP buffer and 3 times with Kinase Reaction Buffer (250mM Hepes, pH 7.2, 50mM $MgCl_2$, 25mM $MnCl_2$, 1mM DTT). Kinase reactions were performed with these HA-immunoprecipitated extracts at 37°C for 30 mins in Kinase Reaction Buffer with 100uM ATP, 10uCi gamma ^{32}P ATP and .5 μg C-terminal GST-Rb (aa769 to 921) (Santa-Cruz biotechnology) as substrate for the reaction. Reactions were stopped by addition of protein sample buffer with 10% betamercaptoethanol and placed on ice. Samples were boiled and separated on 12.5% denaturing acrylamide gel, Coomassie Brilliant Blue stained to ensure equal loading and addition of Rb substrate, and exposed to film overnight.

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TABLES

Table 1: Cell Cycle Effects of cdk4 and cdk6

plasmid transfected	cells counted	Kinase + Brdu +	Kinase + Brdu -
pCMVvector	732	34%	66%
pcmvCDK4	555	35%	65%
pcmvCDK6	902	50%	50%

FIGURE LEGENDS

Figure 1: DNA profiles of vector, cdk6 and cdk4 transfected cells. U2OS cells were transfected with indicated plasmids and harvested for FACS analysis. Cells were incubated with FITC-conjugated CD20 antibody to distinguish transfected populations, fixed and stained with propidium iodine for DNA content. At least 1,800 CD20-positive events were counted per histogram. Results are representative of those found in at least three independent experiments.

Figure 2: DNA profiles of nocodazole treated and untreated cells. U2OS cells were transfected in pairs or remained untransfected, as indicated. At 24 hours after removal of DNA precipitates, the cells were either harvested for FACS or were treated with nocodazole for 18 hours. Results are representative of those found in two independent experiments. At least 2,500 CD20-positive events were counted for cdk4 and cdk6 transfected populations.

Figure 3: Cell cycle phase of transfected cells. A mitotic shake was performed to synchronize transfected U2OS cells. At indicated timepoints after mitotic shake, cells were harvested for either BrdU immunofluorescence (A) or FACS analysis (B). A: Graph of BrdU incorporation of kinase positive cells as determined by immunofluorescence. A minimum of 165 cdk6 positive cells (solid circles) or cdk4 positive cells (open boxes) at each timepoint were scored as BrdU positive or negative. Results are representative of two independent BrdU incorporation experiments. B: DNA profiles of synchronized cells transfected with vector (solid line), cdk6 (dotted line), or cdk6R31C (bold line) and harvested at 4 and 8 hours following mitotic shake and replating.

Figure 4: Biochemical characterization of cdk6 and cdk6 mutants. A: Direct immunoblot of 20 μg of transfected U2OS cell lysate using polyclonal anti-cdk6 antibody C-21. B: cdk6 immunoprecipitation of 400μg transfected cell lysate immunoblotted with polyclonal p18 antibody, then reprobed with cdk6 antibody, as noted. C: cdk6 immunoprecipitation of 200μg U2OS lysate immunoblotted with monoclonal cyclin D1 antibody and cdk6 antibody, as noted. D: cdk6 immunoprecipitation of 400μg cell lysate immunobloted with polyclonal p27 antibody and reprobed with cdk6 antibody, as noted. E: In vitro kinase assay. SAOS-2 cells were transfected with cyclin D1 (lanes 1 and 3-7) or its empty vector (lane 2), and the indicated kinase or its vector. Lysates from each transfection were tested for their kinase activity using C-terminal GST-Rb substrate (aa769-921).

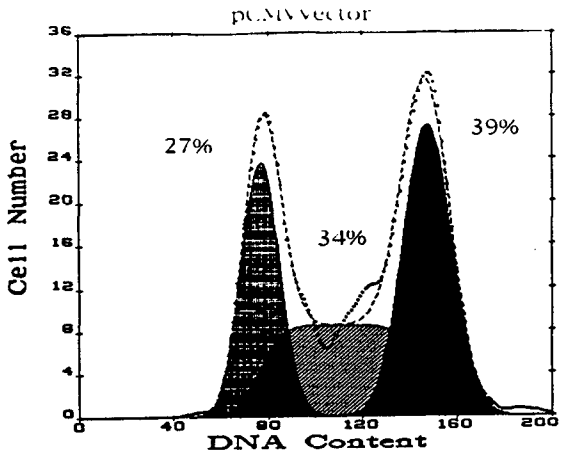
Figure 5: DNA profiles of U2OS cells expressing cdk6 mutants. A: U2OS cells were transfected in pairs and 24 hours after removal of DNA precipitates cells were either harvested for FACS or treated with nocodazole for 18 hours and then harvested for FACS as described previously. At least 3,500 events were counted for each cdk6 mutant histogram, untransfected and p16-transfected controls from this same experiment are shown in figure 2. B: Graph of BrdU incorporation of kinase positive cells as determined by immunofluorescence. BrdU incorporation of transfected U2OS cells harvested at 5, 7, or 10 hours after mitotic shake. At least 120 of vector transfected (closed squares), cdk6wt (closed circles), cdk6R31C (closed triangles), cdk6R31CNFG (open triangles), and cdk6NFG (open squares), positive cells were scored as BrdU positive or BrdU negative at each time point.

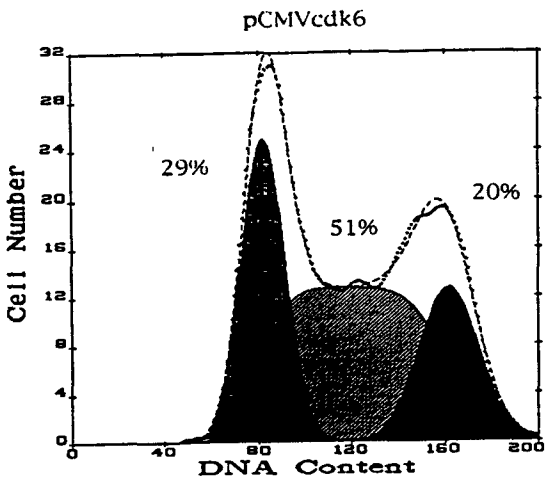
Figure 6: Localization of kinases. Indirect immunofluorescence of transfected cdk6 or cdk6 mutants. A: Cells were fixed at 10 hours after synchronization and stained with polyclonal antibody to cdk6 (C-21). B: Cells were fixed at 9 hours after synchronization and stained with monoclonal antibody to cdk6 (Ab-3).

Figure 7: p18 competes cdk6 from cdc37. Immunoprecipitation of cdk6 from 800μg transfected U2OS cells followed by immunoblot with cdc37 (top panel), and cdk6 (bottom panel). The cdc37 band migrates just below the immunoglobulin heavy chain (*) due to the anti-rabbit immunoprecipitation and anti-rabbit immunoblot.

Figure 8: Speculative model for a positive role of p18 in kinase regulation. After synthesis, newly made cdk6 is stabilized by the complex of hsp90 and cdc37. In this model the INK4 protein, p18, competes cdk6 from the stabilizing hsp90/cdc37complex and retains it in the cytoplasm until the appropriate mitogenic stimulus releases cdk6, possibly through p18 degradation. Release from p18 may also require attack by cyclin D, assembly formation by CIP/KIP (p21/p27) family members, or phosphorylation by a CAK family member. Once complexed with cyclin D, cdk6 is translocated to the nucleus where it may undergo further activation to allow substrate phosphorylation.

PROPRIETARY





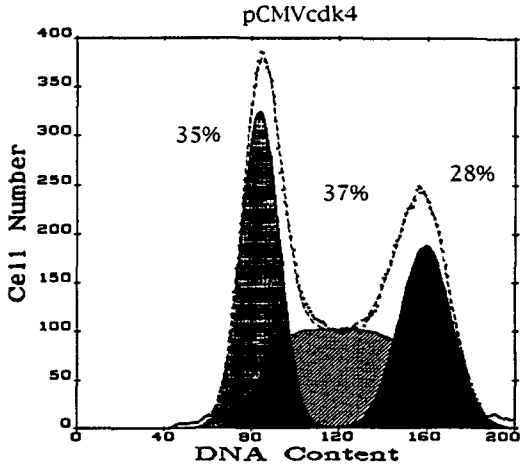


Figure 1

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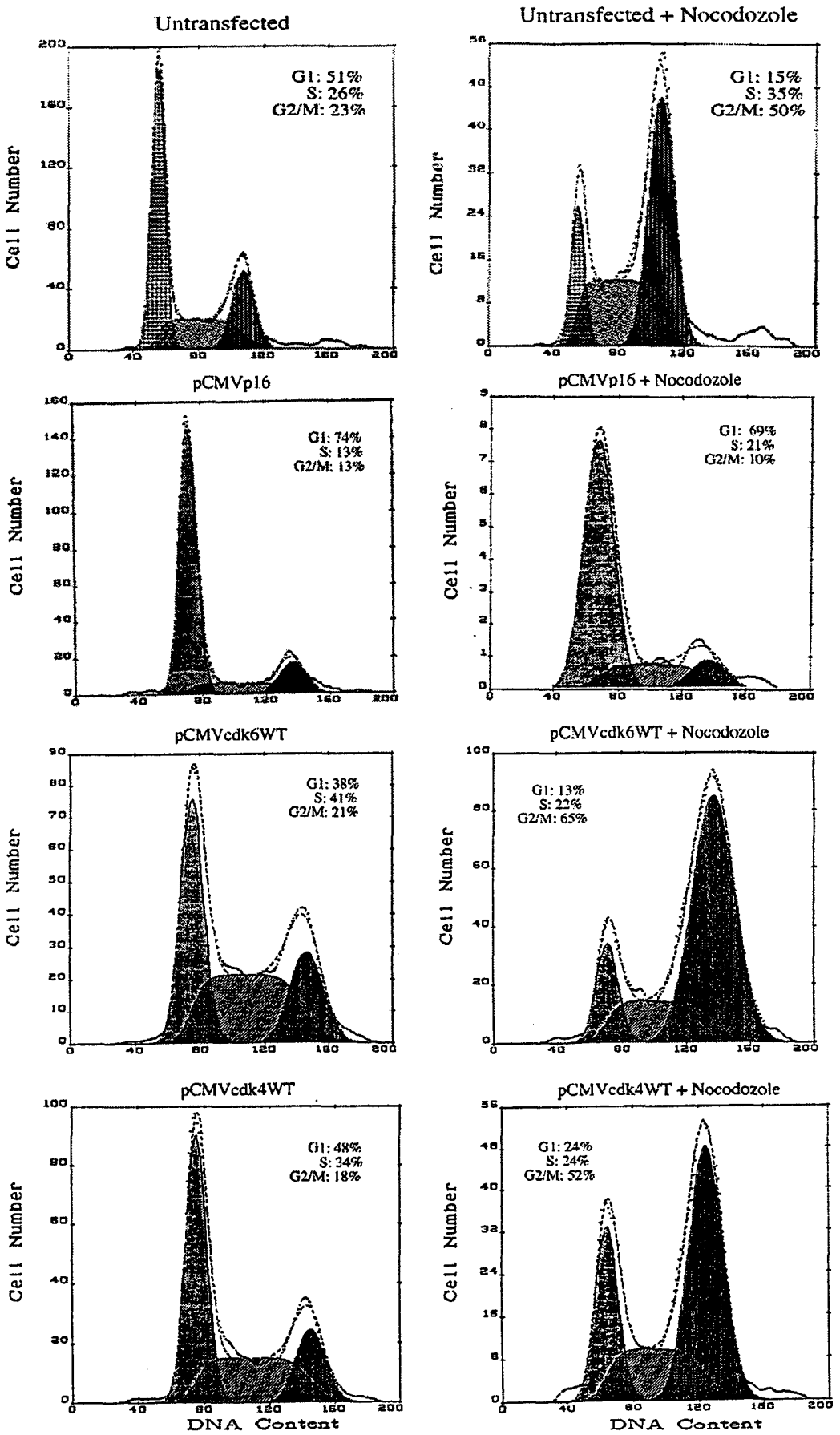

Figure 2

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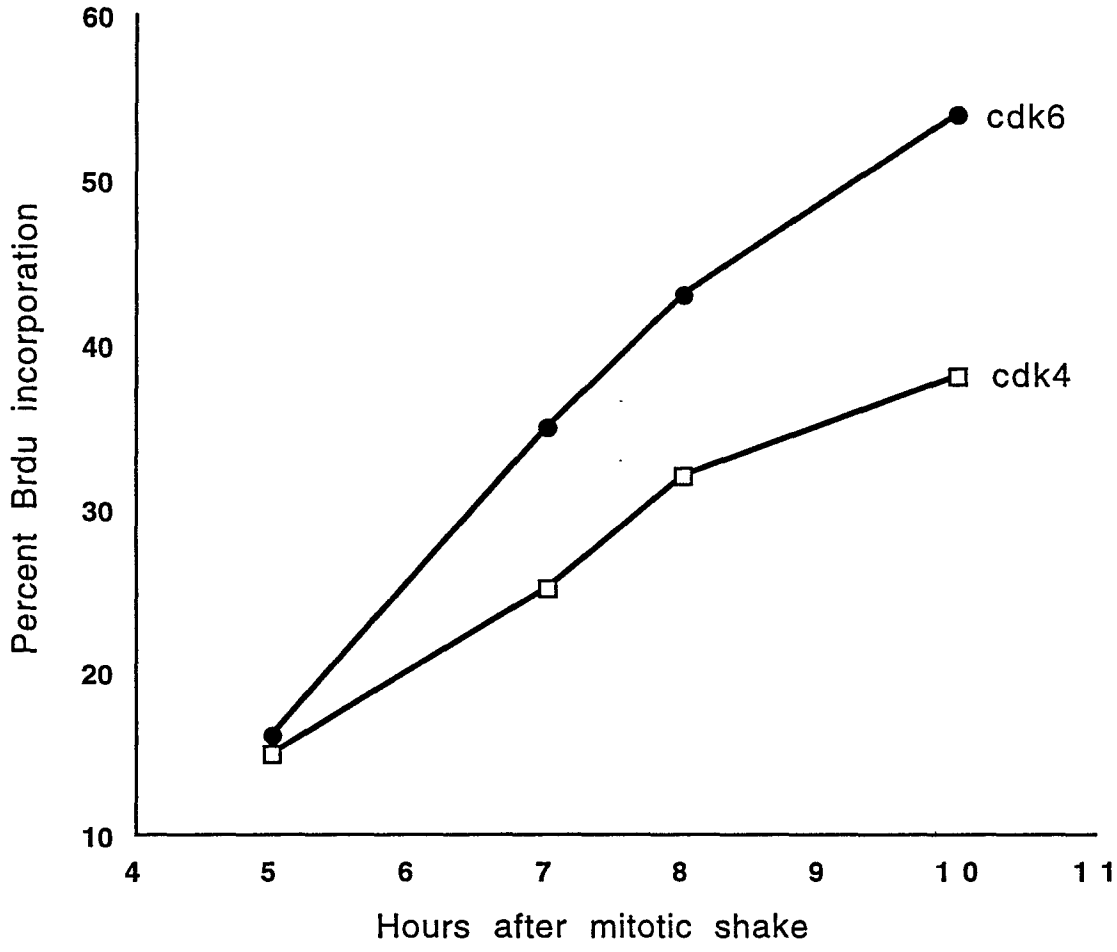
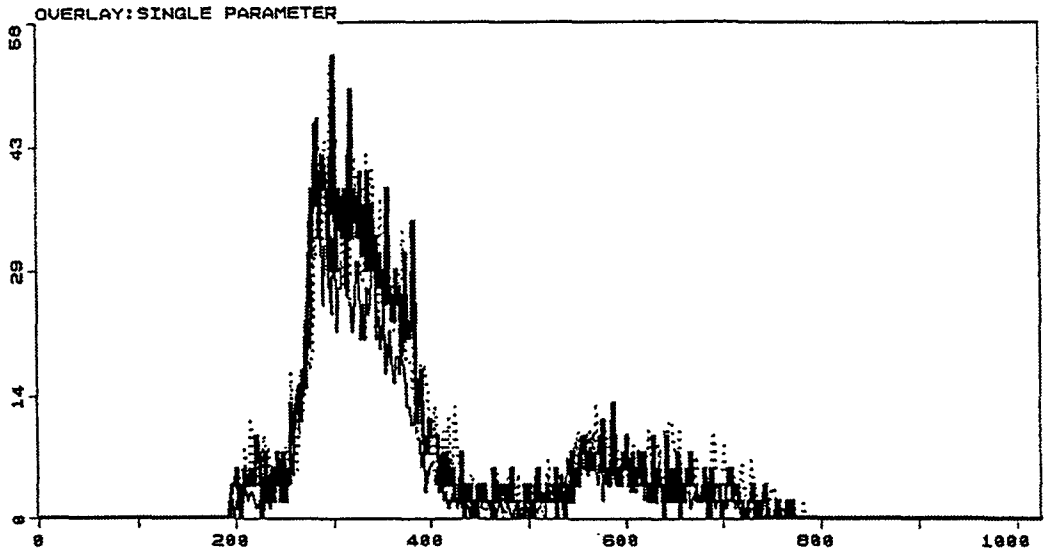


Figure 3A

PROPRIETARY

B

4 hours post Mitotic Shake



8 hours post Mitotic Shake

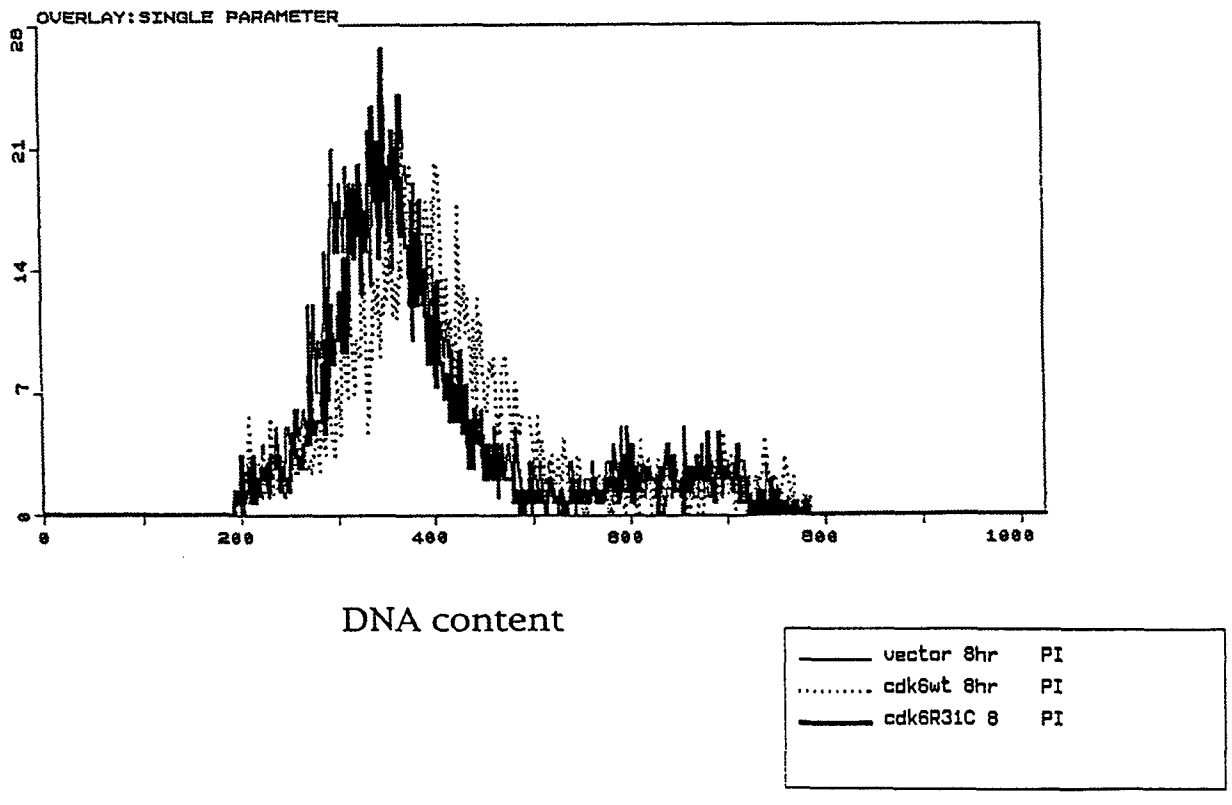
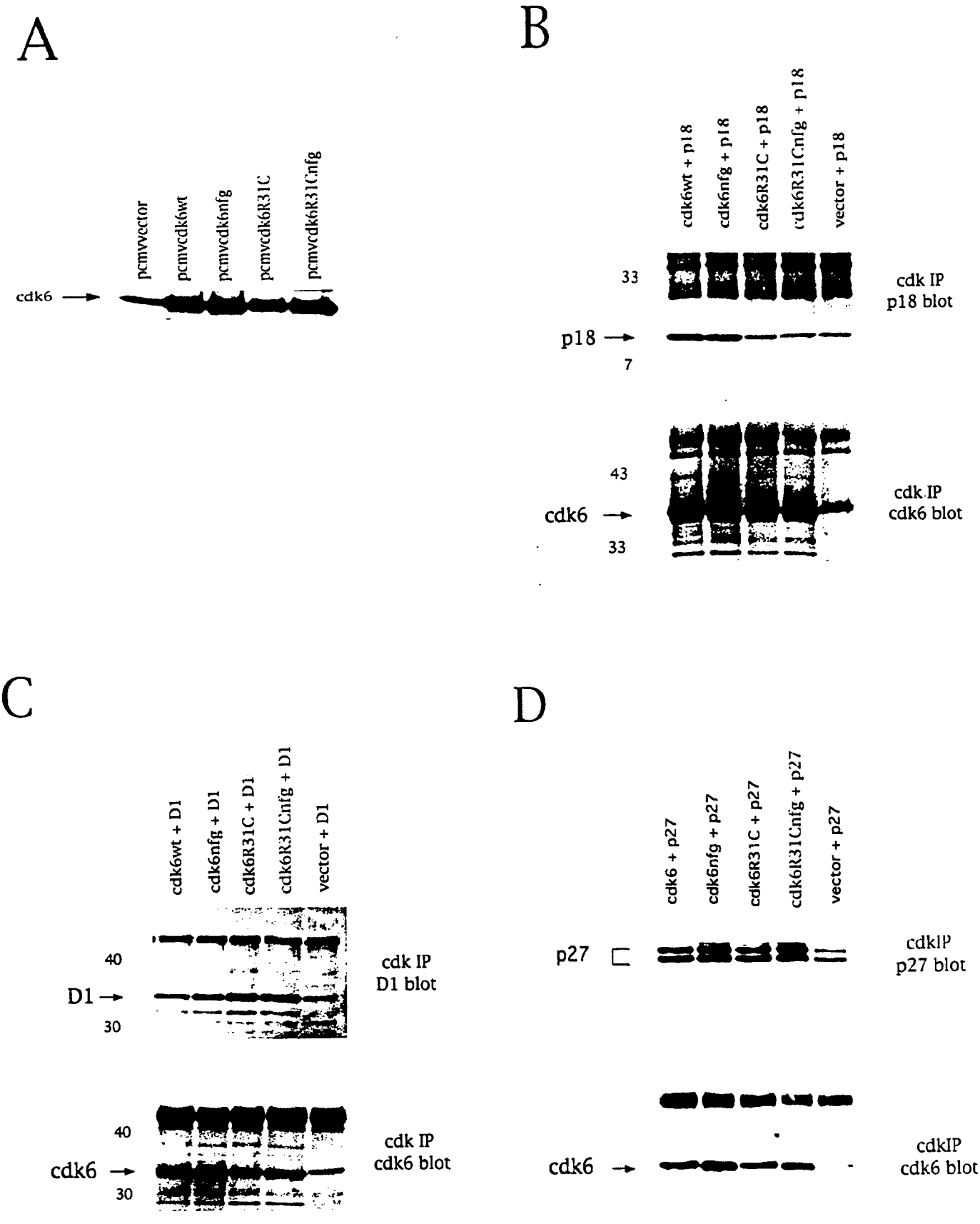


Figure 3B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C
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40
D1
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cdk IP
D1 blot
40
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Figure 4

E

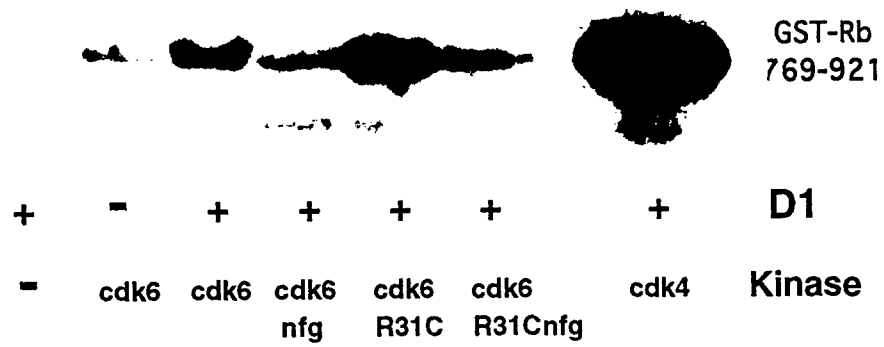

Figure 4 (Continued)

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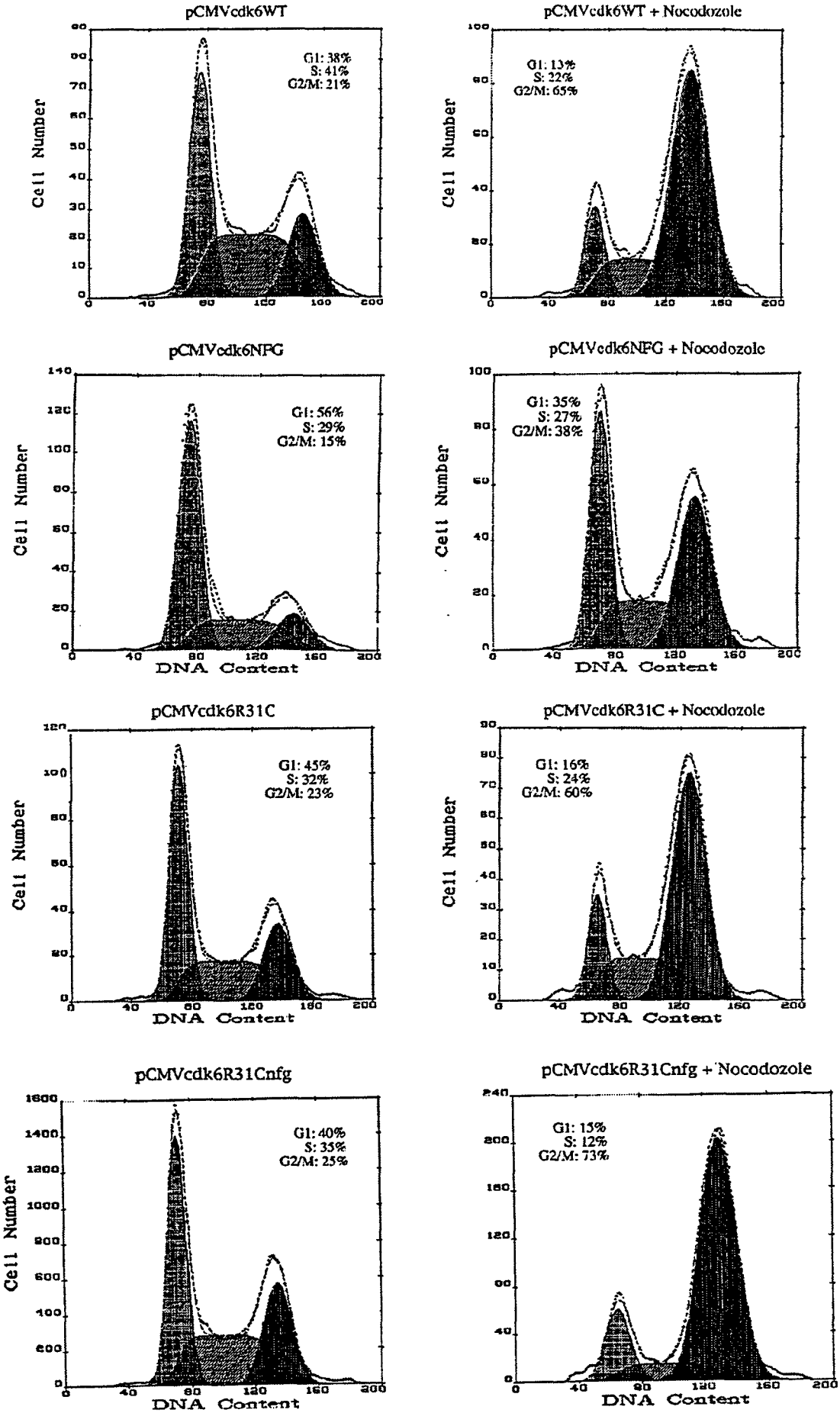


Figure 5A

B

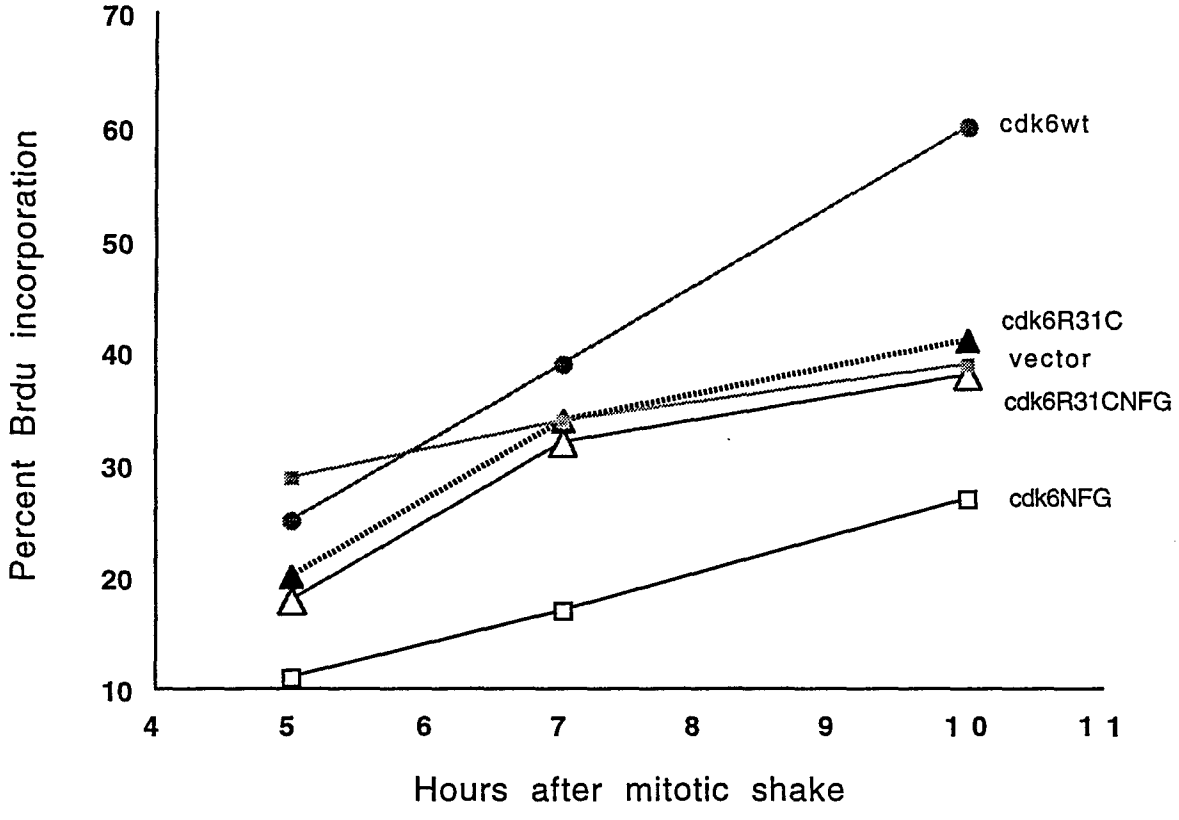
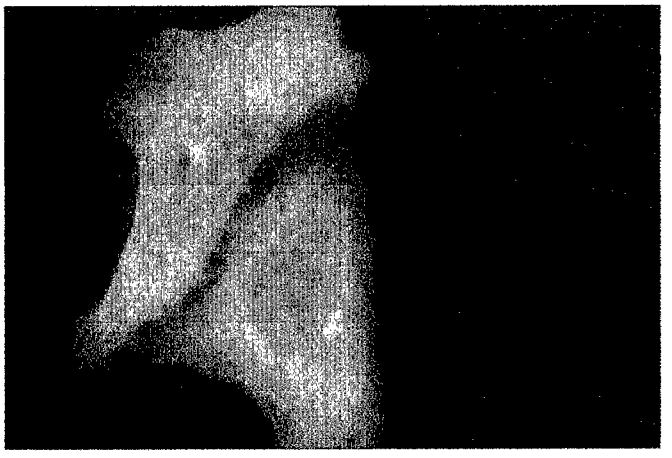


Figure 5B

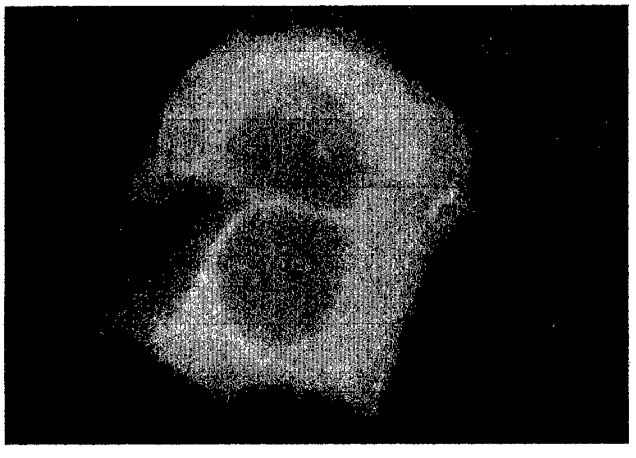
A

Polyclonal anti-cdk6

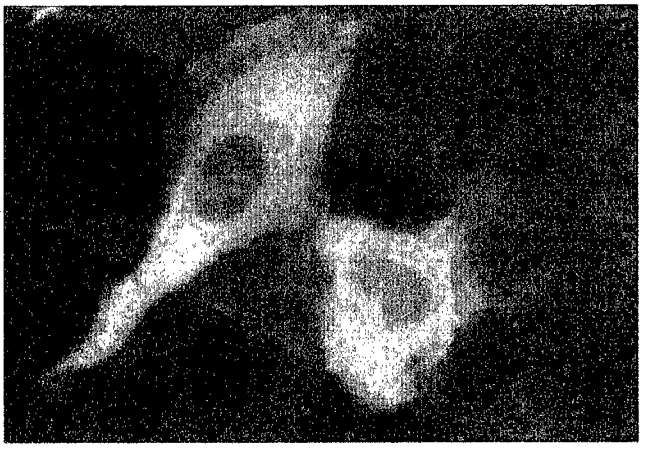
cdk6wt



cdk6NFG



cdk6R31C



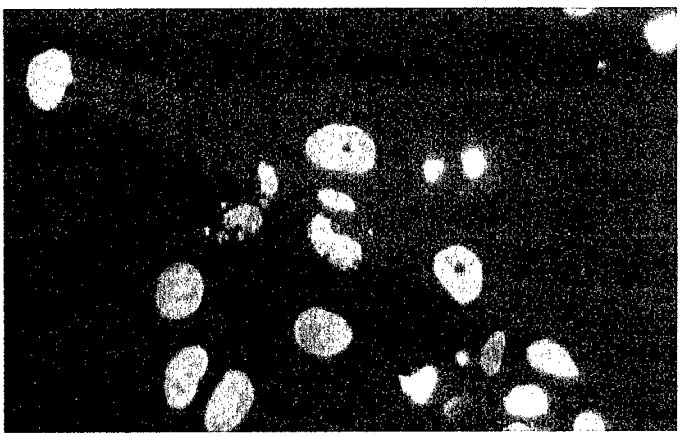
cdk6R31CNFG

Figure 6

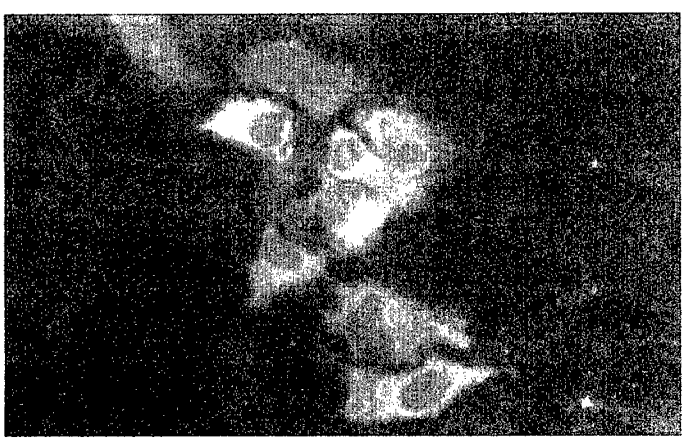
B

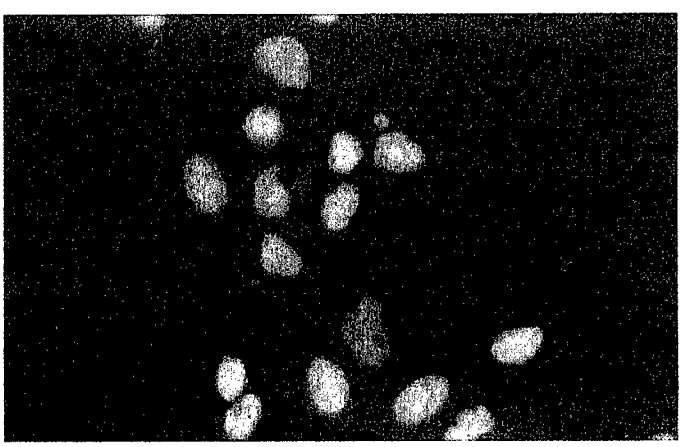
Monoclonal anti-cdk6

Hoechst



cdk6wt





cdk6R31C

Figure 6 (Continued)

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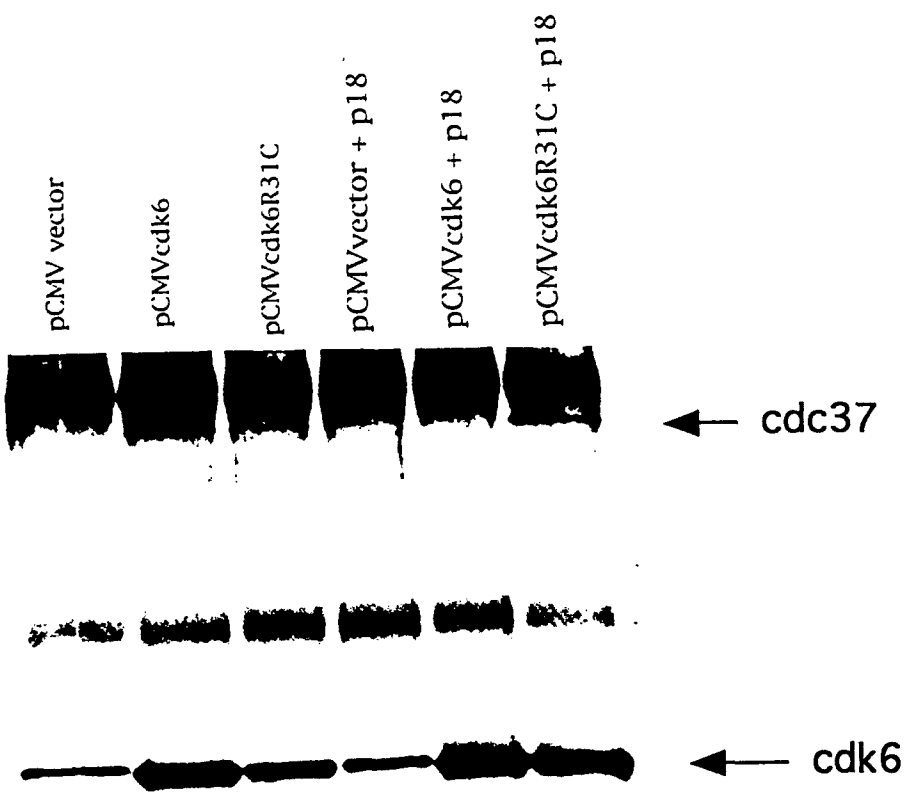

Figure 7

PROPRIETARY

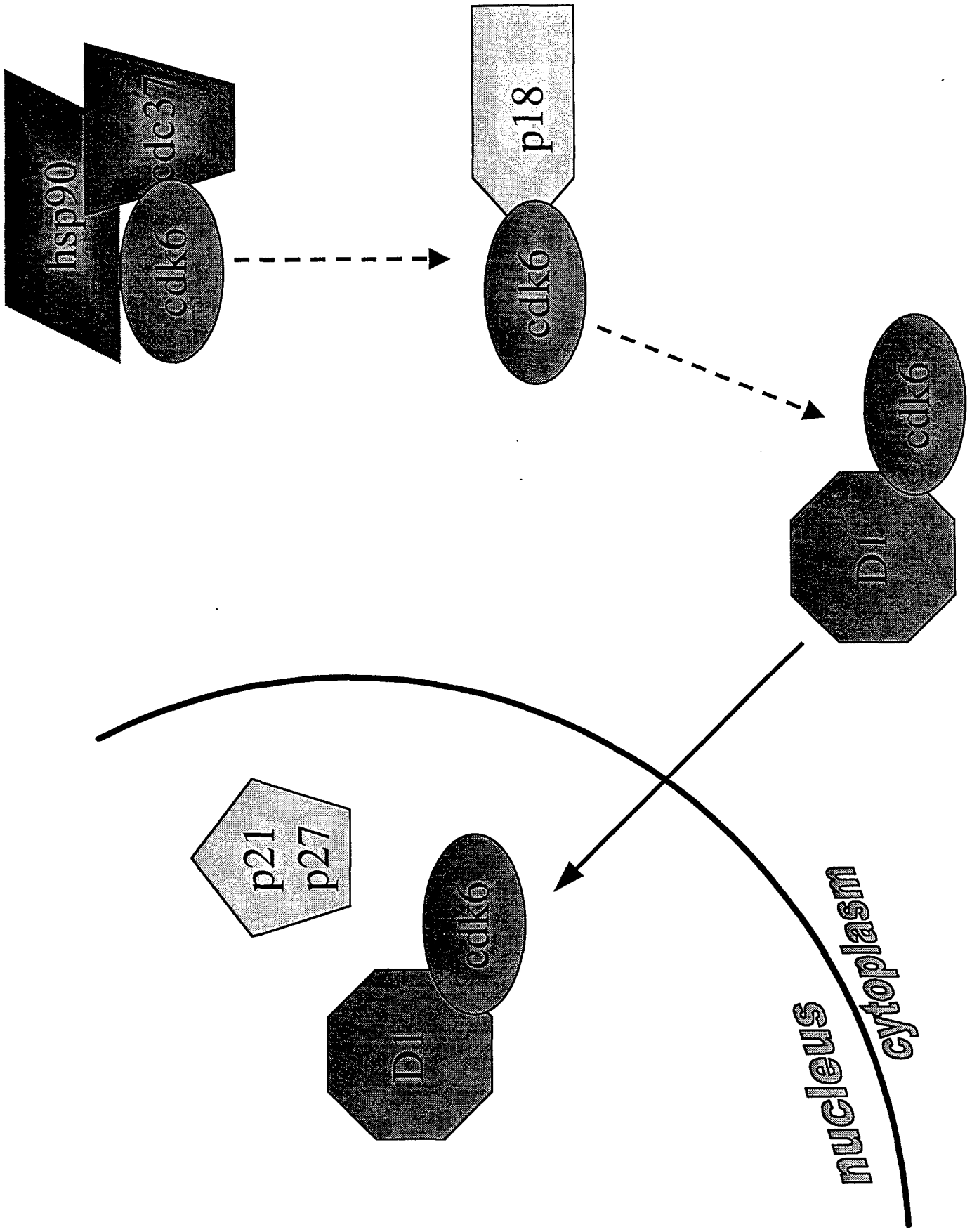

Figure 8

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